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Leptin mRNA Expression in the Mammary Gland of Holstein Dairy Cows

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Abstract: Leptin is secreted mainly from adipocytes, regulates energy metabolism and influences obesity and many other mechanisms. Milk contains higher concentrations of leptin than blood and mammary epithelial cells produce leptin. The present study examines *leptin* gene expression in the mammary glands of lactating and non-lactating cows using RT-PCR. The amplified PCR products were sequenced. Researchers found that unlike the mouse, the leptin mRNA is not expressed at all in the non-lactating mammary gland and is sometimes not expressed in the lactating mammary gland of cows. Sequencing analysis confirmed two leptin precursors like those of human and mouse. One precursor consisted of 167 amino acids and the other consisted of 166 amino acids with a deletion at glutamine 49. This study suggests that leptin may be associated with proliferation of mammary epithelial cells to prepare subsequent lactation during pregnancy.

Key words: Cattle, lactation, leptin, mammary gland, PCR

INTRODUCTION

The *ob* gene was isolated in 1994 as a major factor associated with obesity in the ob/ob mouse (Zhang *et al*., 1994) and the gene product was named leptin. Leptin is a secreted protein that is mainly produced by adipocytes and which regulates food intake and energy expenditure to maintain body fat mass (Auwerx and Staels, 1998; Friedman and Halaas, 1998). Leptin directly acts upon peripheral organs through its own receptors distributed throughout the body and is involved in energy metabolism, reproduction (Chehab *et al*., 1997; McCann *et al*., 1998; Spicer and Francisco, 1997) and the immune system (Lord *et al*., 1998). Leptin is produced not only by adipocyte but also by placenta (Masuzaki *et al*., 1997), stomach (Bado *et al*., 1998) and brain (Wiesner *et al*., 1999). Human milk contains leptin (Casabiell *et al*., 1997; Houseknecht *et al*., 1997) and leptin mRNA is expressed in mammary epithelial cells of humans (Smith-Kirwin *et al*., 1998) and mice (Aoki *et al*., 1999). Leptin mRNA is also detected in bovine mammary epithelial cells (Smith and Sheffield, 2002; Yonekura *et al*., 2006) and leptin can be recovered from cows' milk (Pinotti and Rosi, 2006). However, little is known about leptin in ruminant animals, particularly cattle. Lactogenesis in the mammary glands of ruminants differs from that of single stomach species. Ruminant milk contains a large quantity of saturated short chain fatty acids, only a small amount of which is synthesized from glucose. Leptin should also be quite specific in ruminant animals.

The present study molecularly clones leptin cDNA from the bovine mammary gland and compares leptin mRNA expression in lactating and non-lactating glands.

MATERIALS AND METHODS

Animals and tissue preparations: The 7 lactating and 6 non-lactating Holstein dairy cows that were not pregnant and had calved at least once were slaughtered at a local abattoir. The mammary glands excised and minced under sterile conditions were immediately frozen on dry-ice and stored at -80°C until use. A portion of the tissues was fixed in 10% formalin for histological analysis. This experiment was performed in compliance with the guidelines for the care and use of Laboratory Animals of Azabu University, School of Veterinary Medicine, Sagamihara, Japan.

RNA preparation and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR): Total RNA was isolated using the RNeasy® Mini Kit (Qiagen, Germany) and 1 μg was reverse transcribed using Super Script® II Rnase H Reverse Transcriptase and Oligo $(dT)_{12\text{-}18}$ primer (Life Technologies, USA), according to the manufacturer's instructions. To examine the expression

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of the genes encoding leptin, β-casein (a marker of mammary epithelial cell differentiation) and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), sense and antisense primers were designed on the basis of reported DNA sequences. The primers (each set listed in order of forward and reverse primers) were as follows: Bovine leptin (DDBJ accession number U50365; the bovine leptin primer included sequences from intron 2 and the coding region), 5'-^{1083}CCAGAAGCCCATCCCGG GAAGG^{2004}-3' and 5'-^{3466}CTGGCCTGCATAAAG GATGCCC^{3445}-3'; bovine β-casein (DDBJ accession number M16645), 5'-^{126}CCTGGTGAGATTGTGGAAAG CC^{147}-3' and 5'-^{603}ACTGAGAAAGGGACAGCAC GG^{583}-3' and bovine GAPDH (DDBJ accession number AJ000039), 5'-^{186}CACCATCTTCCAGGAGCGAGAT CC^{209}-3' and 5'-^{761}GACGCCTGCTTCACCACCTTC TTG^{738}-3'. PCR was carried out in a total volume of 50 μL containing 10 mM Tris-HCl (pH 8.0), 50 mM Kcl, 20 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 μM of each primer, 2 U of Ex Taq DNA polymerase (Takara, Tokyo, Japan) and cDNA. Bovine *leptin* gene fragments were amplified by 25 and 35 cycles at 94°C for 40 sec, 58°C for 40 sec and 72°C for 50 sec. The PCR conditions used for bovine β-casein were as follows: 30 cycles at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec; the conditions used for bovine GAPDH were as follows: 30 cycles at 94°C for 40 sec, 64°C for 40 sec and 72°C for 40 sec. The amplified PCR products were separated on a 1.0% agarose gel and visualized by staining with ethidium bromide.

Sequencing of bovine leptin cDNA: The fragments of cDNA amplified by PCR were cloned into the pCRII vector using the TA-Cloning kit Dual Promoter (Invitrogen, USA). Inserts were sequenced using an automatic DNA sequencer (ABI PRISM 310, Perkin-Elmer, USA).

RESULTS AND DISCUSSION

Leptin mRNA expression in bovine mammary gland: Lactating and non-lactating mammary glands were distinguished by the presence of milk exudation and histological analysis. Figure 1 shows the results of the analyses of the expression of mRNAs encoding leptin, β-casein and GAPDH. β-casein mRNA was detected in the mammary glands from all lactating cows and in 1 of the 6 non-lactating cows, suggesting that the definition of lactating and non-lactating for cows was valid. Leptin mRNA expression was detected in the glands of 4 of the 7 lactating cows after 35 cycles of PCR and amplification of leptin mRNA was also detected in glands from 2 cows after 25 cycles of PCR. In contrast to the results for

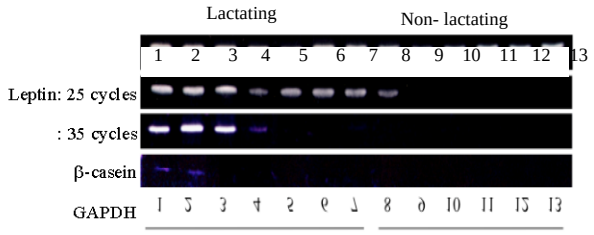

Fig. 1: Expression of leptin, β-casein and GAPDH mRNA in the mammary glands of non-pregnant cows for lactating (lane 1-7) and non-lactating (lane 8-13) mammary glands

lactating cows, the results for leptin mRNA were negative for all 6 non-lactating cows after 25 and 35 cycles of PCR.

Partial sequencing of bovine leptin cDNA from mammary gland: Researchers sequenced two cDNA clones from bovine mammary glands that contained the leptin coding region (Fig. 2). One mammary cDNA clone (Fig. 2, M-1) differed by four base pairs from the reported bovine subcutaneous adipose leptin sequence (Ji *et al*., 1998). Two of them are supposed to produce a change in the corresponding amino acid (CA^{74}G^{75}→CGC:Gln^{25}→Arg) and the other two had no changes in the amino acids (CCT^{69}→CCC:Pro, AAA^{78}→AAG:Lys). Another mammary cDNA clone (Fig. 2, M-2) had six base pairs consisting of silent mutations including two that were identical to those in the M-1 clone (GGT^{396}→GGC:Gly, GTC^{399}→GTT: Val, GCC^{411}→GCT:Ala, CCT^{495}→CCC:Pro). Moreover, a three base pair substitution induced one amino acid alteration (C^{73}A^{74}G^{75}→TGC:Gln^{25}→Cys) and a deletion of three nucleotides caused the absence of the corresponding amino acid residue (C^{145}A^{146}G^{147}→X:Gln^{49}→X) in the M-2 clone.

This study showed that the leptin gene is expressed in some lactating bovine mammary glands and leptin may be secreted from mammary epithelial cells into milk. However, researchers did not detect any expression of the *leptin* gene in non-lactating mammary glands.

Human (Smith-Kirwin *et al*., 1998) and mouse (Aoki *et al*., 1999) mammary epithelial cells produce leptin that is secreted as a part of a fat globule in milk. Lopez-Soriano *et al*. (1998) reported that leptin mRNA levels do not change in lactating rats but litter-removal caused a significant increase in adipose tissue leptin mRNA levels. Moreover, Aoki *et al*. (1999) showed that throughout lactation, leptin mRNA expression in the mouse mammary gland is significantly lower than that of the non-lactating mouse and that leptin down regulation is lactation-dependent in parametrial adipose tissue. Therefore, the expression level of leptin mRNA in

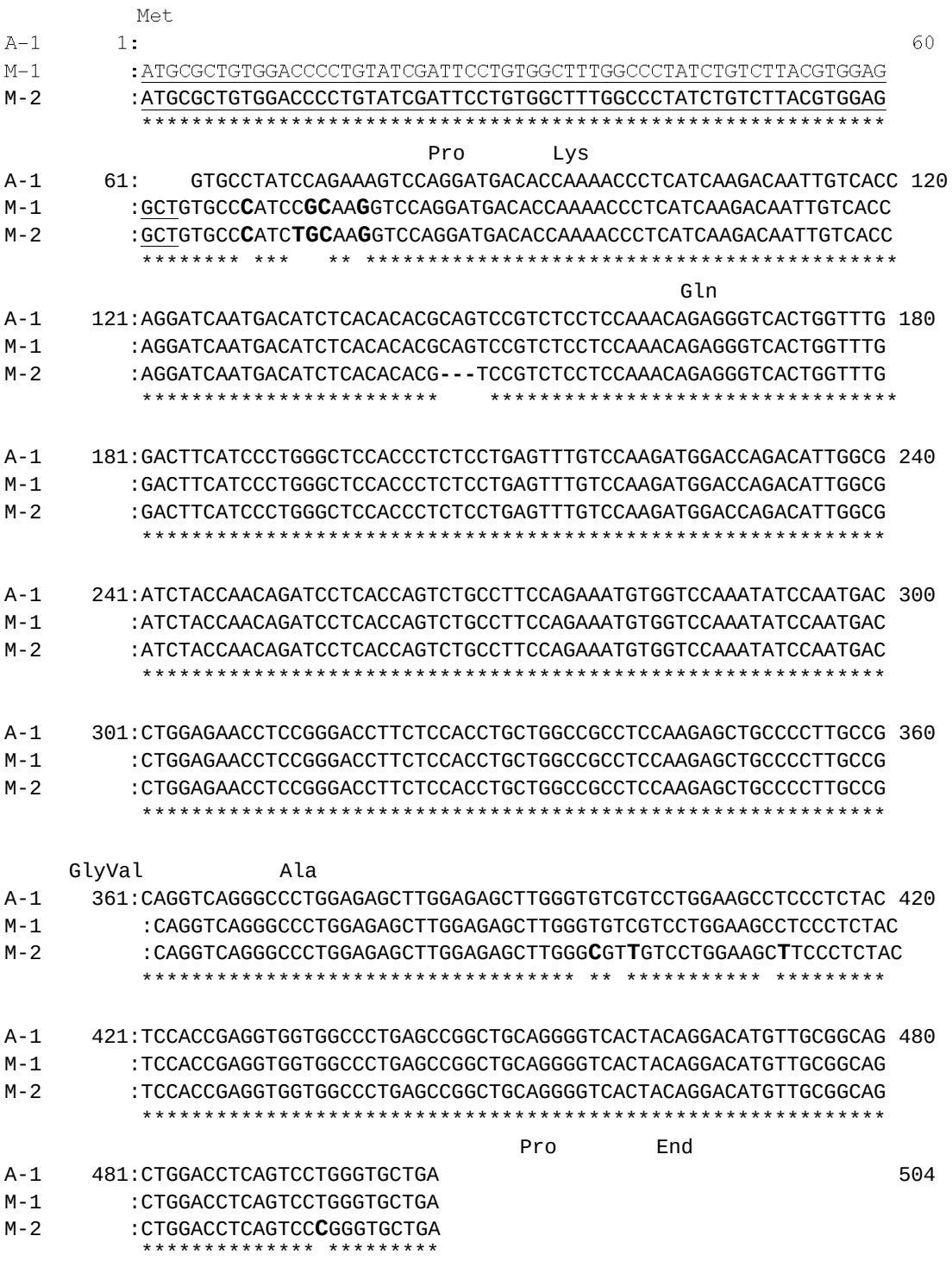

Fig. 2: Comparison of partial bovine leptin cDNA sequences (coding region) in two mammary clones (M-1, M-2) and that of subcutaneous adipose tissue (A-1, DDBJ accession number U65793); signal sequences are underlined and sequence differences are shown in boldface

mammary epithelial cells of post-lactating mouse was restored to pre-lactating levels following the recovery of adipose tissue weight. However, leptin mRNA expression was not identified in the non-lactating mammary glands of

Holstein dairy cows. One of the reasons for this result may be that the amount of body fat does not recover as it does in the mouse. Regardless of pregnancy, *leptin* gene expression in the mouse mammary glands parallels an increase in body fat weight during the post-lactating period but this is not necessarily true in cows. Researchers did not determine the body condition score of all cows but several non-lactating cows were well fattened up in preparation of shipping. Therefore, *leptin* gene expression in the mammary glands appears to have little relation to body fat weight. Another reason may be that all cows in this study were not pregnant. Laud *et al.* (1999) suggested that leptin might be a local growth factor in ovine mammary epithelial cells because the expression of leptin receptor gene increases during mid-pregnancy which is coincident with the initiation of mammary development. Researchers did not address the relationship between pregnancy and leptin expression in this study. We collected mammary tissues from an abattoir. Therefore, information regarding body condition score, age and calving history of the cows was indistinct and could not determine the milk production and lactation stages even in lactating cows. We divided the samples into two groups based only upon the presence or absence of lactation. We plan to obtain more precise profiles of the animals to clarify differences in *leptin* gene expression in the bovine mammary gland. However, the leptin regulation systems of humans, mice and Holstein dairy cows that lactate for over 10 months may differ. Leptin concentrations in the milk of humans (Smith-Kirwin *et al.*, 1998) and mice (Aoki *et al.*, 1999) are higher than those in blood. The presence of leptin in milk might be associated with *leptin* gene expression in the lactating mammary gland. However, whether leptin protein in milk is transferred from blood or produced by mammary epithelial cells, remains to be confirmed. Therefore to understand the presence of leptin in the bovine mammary gland, the results of this experiment need to be examined at the protein level.

Leptin has two variant precursors. One consists of 167 amino acids. The other consists of 166 amino acids lacking a glutamine residue at amino acid position 49. A similar absence of glutamine occurs at a rate of about 30% in humans (Zhang *et al.*, 1994), however this is the first study of this variation in cows.

CONCLUSION

In this study, the difference in *leptin* gene expression in lactating mammary glands may be connected with the long lactation period in Holstein dairy cows. However, the *leptin* gene is not expressed in the non-pregnant and non-lactating mammary gland of cows and thus, leptin may be associated with proliferation of mammary epithelial cells to prepare for subsequent lactation during pregnancy.

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